VEGF RESPONSIVE CELL-BASED ASSAY FOR DETERMINING VEGF BIOACTIVITY

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FIELD OF THE INVENTION

The present invention generally relates to detection of vascular endothelial growth factor (VEGF) in a sample. Specifically, the present invention relates to a VEGF responsive cell-based assay for use in the measurement of the biological activity of VEGF.

BACKGROUND OF THE INVENTION

A number of biological properties have been described for VEGF, including the promotion of angiogenesis. Other properties include endothelial cell migration, endothelial cell proliferation, in vitro capillary tube formation, inhibition of endothelial cell apoptosis, and increased in vivo vascular permeability producing edema.

Because VEGF appears to have a number of significant biological properties, assays for the detection of VEGF and its properties have become increasingly important.

One in vitro bioassay which has been developed is based on the ability of human umbilical vein endothelial cells (HUVEC) to migrate in response to VEGF. This bioassay includes the steps of first virally transfecting Rat-2 cells with the consequent production of VEGF protein, and then testing the produced protein extract for the ability to stimulate HUVEC migration. While this assay can be utilized to demonstrate endothelial cell migration stimulated by VEGF protein in the conditioned media of cells, this assay includes a number of time-consuming steps, which cannot easily be automated.

Recently, it was proposed that VEGF could be a marker of cardiovascular disease risk in patients with hypertension. The measurement of a patient's VEGF and/or FLT-2 levels may be an indicator of the effectiveness of a hypertensive therapy.

Currently, with the seemingly greater importance of VEGF, it would be both advantageous and desirable to have a VEGF responsive assay that is less complex than previous assays, that can be automated, and that is also directly linked to a reporter protein tied to the VEGF receptor (VEGF-R)/FLK-1 signal transduction pathway.

SUMMARY OF THE INVENTION

The present invention provides a method for determining VEGF activity in a sample.

The present invention also relates to a secondary screen which is useful for identifying compounds that modulate VEGF receptors.

The present invention also provides a primary screening mechanism for screening compounds as inhibitors of VEGF function.

The present invention also provides a stable cell line for use in determining VEGF bioactivity and for use in screening compounds which modulate VEGF function or VEGF receptor function.

BRIEF DESCRIPTION OF THE FIGURES

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

Figure 1a is a graph illustrating a VEGF responsive assay wherein the effects of various serum preincubation conditions are shown as a function of increased luciferase expression wherein cells were seeded 24 hours prior to VEGF addition and were harvested 48 hours after VEGF addition;

Figure 1b is a graph illustrating a VEGF responsive assay similar to that shown in Figure 1a wherein the concentration of VEGF utilized in the assay was increased to 50 ng/mL as opposed to 25 ng/mL in Figure 1a;

Figure 2 is a graph illustrating the effects of cell density and $VEGF_{121}$ concentration on luciferase expression in a VEGF receptor stable transfected Hela-Luciferase HLR-ELK-1 cell line;

Figure 3 is a graph illustrating VEGF stimulated production of luciferase in an HLR-ELK-1 cell line wherein the concentration of $VEGF_{121}$ was varied and the number of cells seeded in each well was kept constant at approximately 50,000;

Figure 4 is a graph illustrating VEGF stimulated production of luciferase in a VEGF receptor stable transfected HLR-ELK-1 cell line, wherein the number of cells seeded into each well was kept constant at approximately 50,000, and the concentration of $VEGF_{121}$ was varied;

Figure 5 is a graph illustrating the effect of VEGF antibodies on luciferase production in a VEGF receptor cell line;

Figure 6 is a graph illustrating luciferase production in a VEGF-receptor (VEGFR) cell line wherein incubation time was compared for 24 and 48 hours;

Figure 7 is a graph illustrating the optimal VEGF concentration for stimulation of luciferase production in a VEGF-receptor cell line;

Figure 8 is a graph illustrating the optimal time for VEGF induced luciferase production in a VEGF-receptor cell line;

Figure 9 is a graph illustrating the effect of compound ZD4190 (a known VEGF-receptor tyrosine kinase inhibitor) on VEGF stimulation of luciferase production in a VEGF-receptor cell line;

Figure 10 is a graph illustrating luciferase production after transfection of a VEGF-receptor cell line with $AdVEGF_{121}$; and

Figure 11 is a graph illustrating VEGF stimulated luciferase production using media from $AdVEGF_{121}$ transfected Rat-2 cells.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a VEGF responsive cell-based assay and stable cell line for use therewith for determining VEGF bioactivity. The assay of the present invention allows for the measurement of the bioactivity of VEGF derived from biological samples including plasma, cell culture medium, tissue extracts from tissues or cells transfected with VEGF DNA sequences, or combinations thereof. The method of the present invention can also be adapted for use in high throughput screening and in secondary screens to identify novel small molecule modulators (inhibitors or activators) of a VEGF receptor, specifically FLK-1.

The assay of the present invention utilizes a stable VEGF responsive cell line which comprises HeLa cells which have been stably transfected with a reporter vector having an expressible reporter element and a DNA binding element disposed adjacent thereto. Preferably, the reporter vector includes a gene encoding a detectable gene product which is disposed downstream of a basic promoter element, preferably a TATA box, which is joined to the binding element which is preferably a GAL4 binding element. The stable cell line is also transfected with a vector encoding a CMV promoter-driven ELK-1 transcription factor (which is tied to the MAP kinase pathway) and is fused to a yeast GAL4 DNA binding domain and a yeast GAL4 binding element-driven luciferase reporter construct. A third vector encoding a gene capable of expressing mouse FLK-1 VEGF receptor is also transfected into the cells and the stable cell line generated therefrom can be utilized to demonstrate upregulation for the detectable gene product (luciferase expression) in the presence of VEGF. That is, utilizing established signal transduction pathways, VEGF bioactivity can be assayed.

In general, utilizing known signal transduction relationships and/or pathways, a sample to be assayed for VEGF bioactivity is placed in a container containing the stable cell line as described above. If VEGF is present in the sample, VEGF activates FLK-1 expressed by the stable cell line. Activated FLK-1, which is a known VEGF receptor, then activates MAP kinase (Kroll and Waltenberger, *J. Biol. Chem.*, 1997:272:32521-32527; Doanes et. al., *Biochem. Biophys. Res. Comm.*, 1999;255:545-548. The activated MAP kinase

phosphorylates the fusion trans-activation protein (GAL4 DNA binding domain [dbd] fused with ELK-1). The phosphorylated fusion protein binds to the GAL4 DNA binding site of the reported vector activating luciferase expression. Luciferase expression can be detected utilizing techniques well-known in the art. The presence or expression of luciferase indicates VEGF activity in the sample.

EXAMPLES

The following examples further illustrate the present invention. The examples are intended merely to be illustrative of the present invention and are not to be construed as being limited.

METHODS

Cell Line Production

HeLa cells stably transfected with the GAL4 luciferase reporter and a vector expressing a fusion protein composed of the GAL4 DNA binding domain and the transactivation domain of the transcription factor ELK-1 (GAL4-ELK-1-fusion) were purchased from Stratagene Inc. These cells were co-transfected with a CMV driven FLK-1 expression vector (licensed from The Ludwig Institute, Germany) and a Zeocin selection expression vector (pcDNA3.1/zeo (+), #V860-20) obtained from Invitrogen in the HeLa cells. After appropriate antibiotic selection, stable transfectants were identified that respond to VEGF by increasing luciferase expression.

Cell Culture and Luciferase Assay

The optimal transfected VEGF-receptor cell line (referred to as clone #5) was maintained in Dulbecco's Modified Eagle Medium (Life Technologies, Gaithersburg, MD) containing 10% fetal bovine serum (FBS), Geneticin (250 μg/mL), Hygromysoin B (100 μg/mL), and Zeocin (100 μg/mL). Cells were seeded into 24-well culture plates and allowed to attach prior to the stimulation of luciferase production by the addition of $VEGF_{121}$ (298-VS-005, R&D Systems, Minneapolis, MD) at the indicated concentrations. Luciferase activity was

measured 24 to 48 hours poststimulation as outlined in the technical insert for the luciferase assay system available from Promega Corporation (E1501, Madison, WI). The cells were washed with PBS before being lysed in 200 µL of 1X reporter lysis buffer. The entire plate was frozen at -80°C to increase cell lysis. Upon thawing, 50 µL of the cell extract was transferred into a 96-well plate. Luciferase assay reagent (100 µL) was auto-injected, and light production was measured using a microplate luminometer.

EXAMPLE 1

Effect of Serum Preincubation Conditions on Luciferase Activity

Cells were seeded into wells 24 hours prior to VEGF addition. The cells were harvested 48 hours after the addition of 25 ng/mL of VEGF. Cells were pre-incubated under various serum conditions: (1) 10% FBS, (2) 0.2% FBS, (3) serum free, and (4) nutridoma, a serum-free media supplement. The results are shown in Figure 1a. The optimal serum pre-incubation condition was found to be pre-incubation in 10% FBS. An identified experiment was performed except that the concentration of VEGF added was increased to 50 ng/mL. The results are shown in Figure 1b. The increased concentrations of VEGF was found to increase luciferase expression.

EXAMPLE 2

Effects of Cell Density and $VEGF_{121}$ Concentration on Luciferase Expression

Cells were prepared as described above. Cells were seeded into wells 24 hours prior to the addition of $VEGF_{121}$. $VEGF_{121}$ was added to the cells at concentrations of 25 and 50 ng/mL, respectively. Cells seeded into the wells were tested at densities of: 50, 100, 150, and 200 K/well. The results are shown in Figure 2.

EXAMPLE 3

Dose Response to VEGF in the Parental Cell Line (HLR-ELK-1)

Cells were prepared as described above. 50 K/well were seeded and kept in 10% FBS throughout the experiment. $VEGF_{121}$ was added 24 hours after seeding, and the cells were harvested and analyzed 48 hours after the addition of $VEGF_{121}$. The results are shown in Figure 3. The results showed that the parental cell line was no more responsive to $VEGF_{121}$ stimulation than the control.

EXAMPLE 4

Dose Response of VEGF-Receptor Stable Transfected HLR-ELK1 Cell Line

Cells were prepared as described above. Cells were transfected with the plasmid vector expressing the FLK-1 VEGF receptor were seeded at 50 K/well and maintained in 10% FBS throughout the experiment. $VEGF_{121}$ was added 24 hours after seeding, and the cells were harvested 48 hours after the addition of $VEGF_{121}$. The concentration of $VEGF_{121}$ was tested at 10, 25, 50, 75, 100, and 150 ng/mL. Maximum increase in luciferase expression was found at a $VEGF_{121}$ concentration of 100 ng/mL as shown in Figure 4.

EXAMPLE 5

Dose Responsive Specificity of the VEGF-Receptor Cell Line

To demonstrate the dose responsive specificity of the VEGF-receptor cell line, cells were prepared as described above. The transfected cells were incubated with $VEGF_{121}$, the adenovirus NULL vector (AdCLX), antihuman $VEGF_{121}$ antibodies (AF-293-NA [polyclonal], MAB293 [monoclonal], R&D Systems), a VEGF receptor-specific tyrosine kinase inhibitor (ZD4190, Zeneca [Wedge S.R., Ogilvie D.J. Inhibition of VEGF signal transduction: Identification of ZD4190. *Adv. Exp. Med. Biol.*, 2000;476(Angiogenesis: From the Molecular to Integrative Pharmacology):307-310; and Wedge S.R., Ogilvie D., Dukes M., Kendrew J., Curwen J.O., Hennequin L.F., Thomas A.P., et. al., ZD4190: An orally active inhibitor of vascular endothelial growth factor signaling with broad-spectrum antitumor efficacy. *Cancer Res.*, 2000;60(4):970-975]), media from $ADVEGF_{121}$ transfected rat 2 cells at various concentrations, or the VEGF-receptor cell line

was directly infected with an adenovirus containing $VEGF_{121}$ ($Ad_{GV}121.10$, CI-1023, GenVec, Inc., Rockville, MD) at various concentrations.

Cells were seeded at 50 K/well 24 hours prior to the addition of VEGF/anti-VEGF and were collected 48 hours later. Referring to Figure 5, the results for the antihuman VEGF antibodies are shown. The polyclonal antibodies did not significantly affect luciferase expression. The monoclonal antibodies at the 1:100 and 1:1000 dilutions affected the luciferase expression.

Figure 9 shows the effects of the known VEGF receptor tyrosine kinase inhibitor (ZD4190) on luciferase expression. The VEGF receptor tyrosine kinase inhibitor affected luciferase expression in a dose-response manner.

Figure 10 shows the effects of $AdVEGF_{121}$ obtained from using a media from $AdVEGF_{121}$ transfected rat 2 cells. The addition of $AdVEGF_{121}$ to the VEGF-receptor cell line affected luciferase expression in a close response manner both from the $AdVEGF_{121}$ itself and from the media from $AdVEGF_{121}$ transfected rat 2 cells.

EXAMPLE 6

Luciserase Production in VEGF-Receptor Cell Line at 24 and 48 Hours After the Addition of VEGF

Cells were prepared as described above. $VEGF_{121}$ was added to cells (50 K/well) 24 hours after seeding. $VEGF_{121}$ was added to the cells at concentrations of either 25 or 50 ng/mL. Luciferase expression was measured 24 hours after the addition of $VEGF_{121}$ and 48 hours after the addition of $VEGF_{121}$. The results are shown in Figure 6. Maximum luciferase expression was found in the cells treated with 50 ng/mL of $VEGF_{121}$ at 24 hours post-$VEGF_{121}$ introduction.

EXAMPLE 7

Determination of Optimal VEGF Concentration

VEGF-receptor cells were seeded at 50 K/well and incubated in 10% FBS throughout the experiment. $VEGF_{121}$ was added to the cells 24 hours after

seeding, and the cells were harvested 18 hours later. The $VEGF_{121}$ was applied to the cells at the following concentrations: 1, 5, 10, and 200 ng/mL. The results are shown in Figure 7. The optimal $VEGF_{121}$ concentration ranges from approximately 50 ng/mL to approximately 200 ng/mL.

EXAMPLE 8

Optimal Incubation Time for VEGF-Induced Luciferase Production

Cells were prepared as described above VEGF-receptor cells were seeded at 50 K/well and incubated for 24 hours in 10% FBS prior to the addition of $VEGF_{121}$. Cells were harvested at 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 18, and 24 hours, after $VEGF_{121}$ (25 ng/mL) addition. Figure 8 shows the results of this experiment. Approximately 18 hours of expression time was found to yield maximum luciferase expression (production).

All publications mentioned in the specification are herein incorporated by reference to the same extent as if each independent publication was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. The application is intended to cover any variations, uses, or adaptations following, in general, the principles of the invention and including such departures from the present disclosure within known or customary practice within the art to which the invention pertains and may be applied to the essential features herein before set forth.